MP65-05: NOTCH2 in the bladder promotes tumor development and leads to more malignant phenotype.

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ABSTRACT

Introduction: Our recently published results revealed that NOTCH2 is an oncogene that drives bladder cancer (BCa) progression. To test whether NOTCH2 can promote BCa development, we have established a constitutively active NOTCH2 intracellular domain (N2ICD) mouse model. Previous TCGA data showed that NOTCH2 expression and copy number gain are enriched in basal tumors.

Methods: We have created lentiviral constructs based on the FUGW vector containing the N2ICD transgene, driven by either uroplakin-2 (Upk2 for luminal) or cytokeratin-5 (Krt5 for basal). These constructs also included intraribosomal entry sequence (IRES) followed by the firefly luciferase gene. Lentiviral particles were inoculated by ultra-sound guided injections into the subepithelial space of the bladder wall. Mice were treated with or without N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN; bladder specific carcinogen). We have also tested the metastatic potential of NOTCH2 mutants (A2025E and Q2223X) over-expressed cell lines in vivo using the zebrafish embryo model. Each wild-type, NOTCH2 mutants (A2025E and Q2223X), N2ICD over-expressed RT4 or RT112 cell lines, and NOTCH2 knock-down UC13 cell lines were injected to zebrafish embryo. After 3 days, the number of metastasis was counted.

Results: NOTCH2 over-expression by lentiviral infection was observed in vitro. Tumorigenesis was observed in some mice. There were 2 cases in mock group (2/15, 13.3%), 6 cases in the Upk2 group (6/15, 40%), and 5 cases in Krt5 group (5/15, 33.3%). The tumors in both Upk2 and Krt5 group showed not only urothelial carcinoma but also squamous cell carcinoma features. Moreover, Tumorigenesis in both Upk2 and Krt5 group was observed at week 20 with BBN treatment, whereas at week 26 in mock group. In the zebrafish model, the number of metastasis was increased in the N2ICD over-expressed groups compared to the wild-type and mutant lines. On the other hand, the metastasis was decreased in NOTCH2 knock-down UC13 line.

Conclusion: Our results suggest that the over-expression of N2ICD in the bladder wall could accelerate the tumor development and possibly lead to more malignant phenotypes. Moreover, the over-expression of N2ICD promoted metastatic potential.

BACKGROUND

- Notch is a family of four cell surface receptors (Notch 1-4) that regulates differentiation, proliferation, and invasion (Figure 1).
- In bladder cancer (BCa), Notch1 (N1) acts as a tumor suppressor (Rampias et al. 2014, Maraver et al 2015), whereas Notch2 (N2) acts as an oncogene (Hayashi et al. 2016) (Table 1).
- Increased N2 signalling is featured in basal tumors (TCGA).

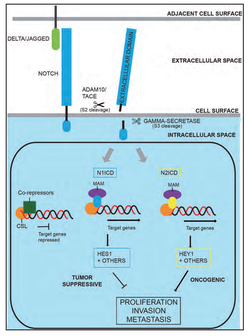


Figure 1: The core Notch pathway in bladder cancer.

The core Notch pathway in bladder cancer.
Binding of the Delta ligand (light green) on one cell to the Notch receptor (blue) on another cell results in two proteolytic cleavages of the receptor. The ADAM10 (a disintegrin and metalloproteinase 10) or TNF-alpha-converting enzyme (TACE) metalloprotease catalyses S2 cleavage, generating a substrate for S3 cleavage by the gamma secretase complex. This proteolytic processing mediates release of the Notch intracellular domain (NICD), which enters the nucleus and interacts with the DNA binding CSL (Cbf1, Su(H), Lag1) (orange).
The co-activator mastermind (MAM; purple) and other transcription factors (blue and yellow) are recruited to the CSL complex, whereas co-repressors (green) are released.
(Goriki et al. 2018)

OBJECTIVES

- To demonstrate the ability of N2 to induce BCa in a mouse model of bladder-specific over-expression of N2.
- To delineate the impact of Notch2 mutations on metastasis.

Table 1: Dual role of NOTCH in cancer.

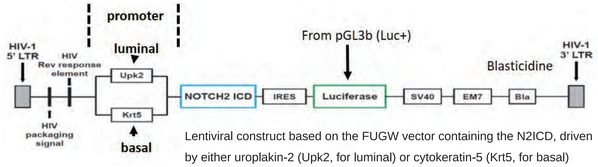
Tumor type	NOTCH type	Role of Notch signaling	Putative or observed effect	References
T-ALL	NOTCH1	Oncogene	Activating NOTCH1 mutation induces ligand independent activation	Ellisen et al., 1991
	NOTCH3	Oncogene	Activating NOTCH1 mutation increases stability of N1ICD	Weng et al., 2004
			Activating NOTCH3 mutation increases ligand-independent NOTCH3 activation	Malyukova et al., 2007
				Thompson et al., 2007
				Masser et al., 2007
				O'Neil et al., 2007
				Bernasconi-Elias et al., 2016
CLL	NOTCH1	Oncogene	Activating NOTCH1 mutation increases stability of N1ICD	Fabbri et al., 2011
	NOTCH2	Oncogene	Activating NOTCH1 mutations are associated with poor prognosis	Puente et al., 2011
			Inactivating NOTCH1 or NOTCH2 increases apoptosis of CLL cells	De Falco et al., 2015
				Willander et al., 2013
NSCLC	NOTCH1	Oncogene	NOTCH1 activation is associated with poor prognosis	Westhoff et al., 2009
	NOTCH2	Tumor suppressor	Inactivating NOTCH2 increases carcinogenesis in NSCLC	Baumgart et al., 2015
PDAC	NOTCH1	Tumor suppressor	Inactivating NOTCH1 are associated with PDAC incidence and progression	Hanlon et al., 2010
	NOTCH2	Oncogene	activating NOTCH2 induces progression and correlated with poor survival	Mazur et al., 2010
HCC		Tumor suppressor	Enforced activation of Notch1 induces cell cycle arrest and apoptosis	Viatour et al., 2011
	NOTCH1		Activated Notch-related gene (HES1) correlated with better survival	Qi et al., 2003
		Oncogene	Inactivating NOTCH1 decreases the migration and invasion	Zhou et al., 2013
				Cantarini et al., 2006
	NOTCH2	Oncogene	Activation of NOTCH2 increases the migration and invasion	Hayashi et al., 2015
CMML	NOTCH1	Tumor suppressor	Inactivating Notch pathway are associated with the development of CMML	Klinakis et al., 2011
	NOTCH2	Tumor suppressor	CMML patients posess loss of function mutation in NOTCH2	
HNSCC	NOTCH1	Tumor suppressor	Truncated or ligand-binding inefficient receptors	Stransky et al., 2011
	NOTCH2	Tumor suppressor	Predicted to impair differentiation	Agrawal et al., 2011
	NOTCH3	Tumor suppressor	HNSCC patients posess loss of function mutations in NOTCH1,2, and 3	Pickering et al., 2013
	NOTCH1	Oncogene	Overexpression of NOTCH1 and 2 is observed in HNSCC	Leethanakul et al., 2000
	NOTCH2	Oncogene	Overexpression of NOTCH1 and 2 is observed in HNSCC	Hijioka et al., 2010
	NOTCH3	Oncogene	Overexpression of NOTCH1 and 3 is observed in HNSCC	Zhang et al., 2011
				Yoshida et al., 2013
				Sun et al., 2014
B-ALL	NOTCH1	Tumor suppressor	Activation of NOTCH1-4 induce B-cell growth arrest and apoptosis	Zweidler-Mckay et al., 2005
	NOTCH2	Tumor suppressor		
	NOTCH3	Tumor suppressor	NOTCH3 is hypermethylated in B-ALL	Kuang et al., 2013
	NOTCH4	Tumor suppressor		
MB	NOTCH1	Tumor suppressor	NOTCH1 activity inhibits proliferation	Fan et al., 2004
	NOTCH2	Oncogene	NOTCH2 promotes cell growth	
BCA	NOTCH1	Tumor suppressor	NOTCH pathway inactivation prormotes bladder cancer	Greife et al., 2014 Rampias et al., 2014 Maraver et al., 2015
	NOTCH2	Oncogene	Forced overexpression of N2ICD induced cell growth and invasion	Hayashi et al., 2016
	NOTCH3	Oncogene	NOTCH3 overexpression promotes cell growth and chemoresistance	Zhang et al., 2017
Breast Ca	NOTCH1	Oncogene	Increased expression of NOTCH1 correlates with poor prognosis	Reedijk et al., 2005
	NOTCH3	Oncogene	Overexpression of NOTCH1,3 and 4 induces mammary tumors	Hu et al., 2006
	NOTCH4	Oncogene	Inactivating NOTCH4 reduces tumorigenic potentioal	Harrison et al., 2010
				Gallahan et al., 1996
Skin cancer	NOTCH1	Tumor suppressor	NOTCH1 activation induces differentiation and cell cycle arrest	Lowell et al., 2000
			Deletion of NOTCH1 increase the basal epidermal layer	Rangarajan et al., 2001
				Nguyen et al., 2006
Melanoma	NOTCH1	Oncogene	NOTCH1 is overexpressed in Melanoma	Bedogni et al., 2008
			NOTCH1 converts primary melanoma cells from noninvasive to metastatic	Liu et al., 2006

T-ALL: T cell acute lymphoblastic leukemia, CLL: chronic lymphocytic leukemia, HSCLC: non-small cell lung carcinoma, PDAC: pancreatic ductal adenocarcinoma, HCC: hepatocellular carcinoma, CMML: chronic myelomonocytic leukemia, HNSCC: head and neck squamous cell carcinoma, B-ALL: B cell acute lymphoblastic leukemia, MB: medulloblastoma, BCA: bladder cancer.

Notch signalling has been implicated in various solid tumors, and different Notch receptors have been shown to have disparate roles in cancer development and progression. (Goriki et al. 2018)

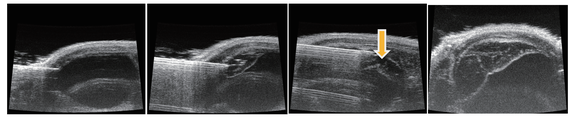
METHODS

Figure 2: N2ICD lentiviral construct generation.



Lentiviral construct based on the FUGW vector containing the N2ICD, driven by either uroplakin-2 (Upk2, for luminal) or cytokeratin-5 (Krt5, for basal)

Figure 3: Lentivirus injection.

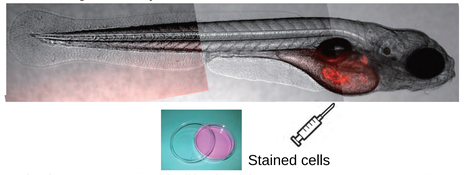


Lentiviral particles were inoculated by ultra-sound guided injection into the subepithelial space of bladder wall (Nude mouse).

Figure 4: Inoculation schedule.

Each group has 15 mock, 15 Upk2-N2ICD, and 15 Krt5-N2ICD mice. Mice in Group 1 received only lentivirus inoculation. Mice in Group 2 and 3 received lentivirus inoculation, then they were treated with 0.05% BBN for 8 weeks (Groiup 2) or 12 weeks (Group 3). Sampling started at week 20 and 3 mice were sacrificed every 2 weeks.

Figure 5: Zebrafish xenograft embryo model.

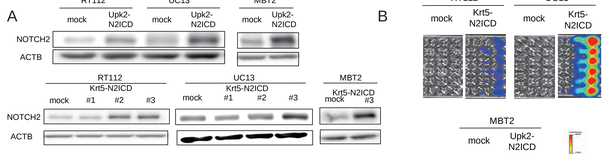


Stained cells

Cells were labeled with Vybrant CM-DiI cell labeling solution prior to injection in the zebrafish embryo.
Fluorescent microscopy for identification of metastasis was conducted 3days later.

RESULTS

Figure 6: N2ICD over-ezpression *in vitro*.



A: Lentivitral constructs (Upk2-N2ICD or Krt5-N2ICD) were driven in bladder tumor cell lines (RT112, UC13, and MBT2).
B: Cell lines infected with Upk2-N2ICD or Krt5-N2ICD had luciferase activity.

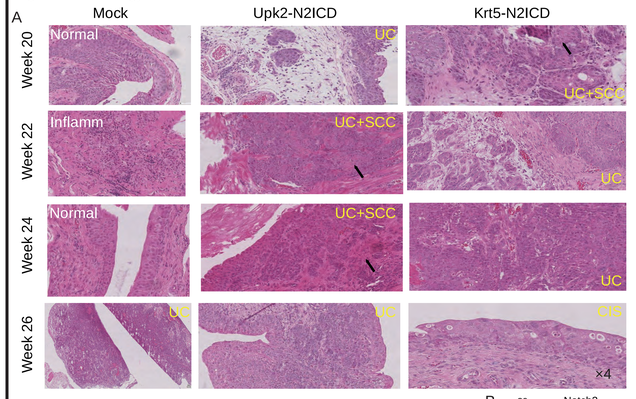
Figure 7: The result of lentivirus inoculated mouse model.

	Tumorigenesis
Group1	no spontaneous tumors at week 48
Group2	0/5 (0%) mock
	0/5 (0%) Upk2-N2ICD
	2/5 (40%) Krt5-N2ICD

	Tumorigenesis	Bladder weight (g)	Bladder tumor histopathology
Group3	2/15 (13.3%) mock	33.4±12.2	UC (2/15)
	6/15 (40%) Upk2-N2ICD	47.9±27.7	UC (4/15), UC+SCC (2/15)
	5/15 (33.3%) Krt5-N2ICD	53.7±68.5	UC (2/15), UC+SCC (3/15)

There was no spontaneous tumors in Group 1 at week 48. In Group 2, there were 2 bladder cancer cases (Bca) in Krt5-N2ICD over-expressed mice. In Group 3, mock mice had 2 BCa cases, Upk2-N2ICD mice had 6 BCa cases, and Krt5-N2ICD mice had 5 BCa cases.

Figure 8: Histology of the bladder of mice in Group 3.



A: Histology of the bladder of mice in Group 3 (H&E stained). Tumorigenesis in mock mice was observed at week 26, while at week 20 in Upk2-N2ICD and Krt5-N2ICD over-expressed mice. Some tumors have squamous cell differentiation(arrow).
B: mRNA expression of Notch2 in mouse bladder tissue. The relative levels of mRNA were normalized to the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The mRNA level of mock was set to 1.

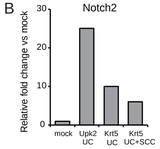
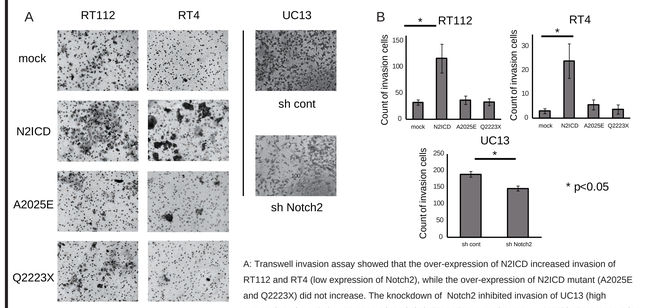
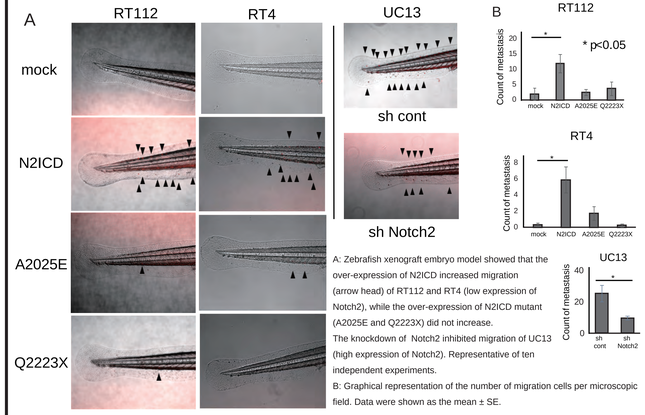


Figure 9: N2ICD over-expression increased invasion.



A: Transwell invasion assay showed that the over-expression of N2ICD increased invasion of RT112 and RT4 (low expression of Notch2), while the over-expression of N2ICD mutant (A2025E and Q2223X) did not increase. The knockdown of Notch2 inhibited invasion of UC13 (high expression of Notch2). Representative of six independent experiments. B: Graphical representation of the number of invasive cells per microscopic field. Data were shown as the mean ± SE.

Figure 10: N2ICD over-expression increased migration.



A: Zebrafish xenograft embryo model showed that the over-expression of N2ICD increased migration (arrow head) of RT112 and RT4 (low expression of Notch2), while the over-expression of N2ICD mutant (A2025E and Q2223X) did not increase.
The knockdown of Notch2 inhibited migration of UC13 (high expression of Notch2). Representative of ten independent experiments.
B: Graphical representation of the number of migration cells per microscopic field. Data were shown as the mean ± SE.

CONCLUSIONS

- Overexpression of N2ICD under luminal/basal promoters was not sufficient to induce BCa tumorigenesis.
- N2 overexpression might help accelarate BCa development under both luminal and basal promoters in the BBN-induced BCa mouse model.
- Luminal and basal N2-tumors showed UC and sometimes SCC features.
- Over-expression of N2ICD increased cell invasion and migration, while the over-expression of N2ICD mutants did not increase. Moreover, Notch2 knock-down decreased cell invasion and migration.